3α-, 7α-, and 12α-OH group specific enzymic analysis of biliary bile acids: comparison with gas–liquid chromatography

Ian A. Macdonald, C. Noel Williams, and Bonnie C. Musial

Department of Medicine, Dalhousie University, Halifax, N.S., Canada

Summary 3α-Hydroxysteroid dehydrogenase (3α-HSDH) from *P. testosteroni,* 7α-HSDH (*Escherichia coli* ATCC #29532) and 12α-HSDH (*Clostridium group P,* strain C48-50, ATCC #29733) were used to directly measure 3α-, 7α-, and 12α-OH groups in extracted human bile-rich duodenal aspirates. Twelve samples chosen for widely differing ratios of cholic/chenodeoxycholic/deoxycholic were computed by solving three simultaneous equations. Comparison of these ratios with those obtained by *a*) thin-layer chromatography and 3α- and 7α-HSDH assays and *b*) gas–liquid chromatographic analysis showed no significant difference. Addition of known amounts of pure cholic, chenodeoxycholic, deoxycholic, or lithocholic acid to individual bile extracts gave an appropriate yield of 3α-, 7α-, and 12α-OH groups. The direct (non-chromatographic) enzymic method has the advantages of being rapid, convenient, and inexpensive, and thus suitable for clinical use.—**Macdonald, I. A., C. N. Williams, and B. C. Musial.** 3α-, 7α-, and 12c4a-OH group specific enzymic analysis of biliary bile acids: comparison with gas–liquid chromatography. *J. Lipid Res.* 1979. **20:** 381–385.

Abbreviations: C, cholic acid; CDC, chenodeoxycholic acid; DC, deoxycholic acid; LC, lithocholic acid, NAD, β-nicotinamide adenine dinucleotide; NADP, β-nicotinamide adenine dinucleotide phosphate; HSDH, hydroxysteroid dehydrogenase; GLC, gas–liquid chromatography; TLC, thin-layer chromatography.

Supplementary key words 3α-, 7α-, and 12α-hydroxysteroid dehydrogenases · hepatofluorobutyrates

Individual bile acids can be quantified by gas–liquid chromatography (1–4) and separated by thin-layer chromatography (TLC). Thin-layer chromatography is usually combined with enzymic or chemical methods to measure individual bile acids (5, 6). The disadvantage of TLC is the failure to separate bile acid components such as the glyco- and tauro-conjugates of isomeric, α-dihydroxy-5β-cholanoates. Additionally, TLC requires preparative cleansing, and the elution of bile salts from silica is incomplete.

In this communication we report the direct, non-

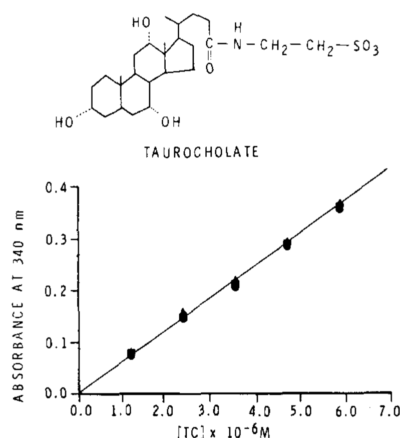


Fig. 1. Standard curve for 3α-HSDH (closed circles, ●——●), 7α-HSDH (closed triangles, ▲——▲), and 12α-HSDH (closed squares, ■——■) using taurocholate as substrate. Assays were performed in duplicate.

Downloaded from www.jlr.org by guest, on June 19, 2012

ASBMB JOURNAL OF LIPID RESEARCH

TABLE 1. Comparison of the ratios of cholic/chenodeoxycholic/deoxycholic acids and total bile acid concentrations by direct enzymic assays (a), gas–liquid chromatography (b), and thin-layer chromatography and 3α- and 7α-HSDH assays (c)

Sample[a]		Percentage Composition			
		Cholic	Chenodeoxycholic	Deoxycholic	Total
					mM
	a	49.2	39.6	11.2	41.5
1	b	52.0	34.0	14.0	32.0
	c	54.6	32.8	12.6	46.8
	a	50.0	44.8	5.2	65.3
2	b	46.0	44.0	10.0	81.0
	c	54.2	39.5	6.3	74.3
	a	45.7	20.2	34.1	37.0
3	b	42.0	23.0	35.0	25.0
	c	39.3	25.0	35.7	34.0
	a	28.8	25.7	46.5	46.0
4	b	23.0	28.0	49.0	43.0
	c	26.6	25.2	48.2	43.6
	a	55.1	31.9	13.0	34.0
5	b	58.0	28.0	14.0	41.0
	c	60.6	26.6	12.8	38.8
	a	53.0	41.8	6.2	45.0
6	b	54.0	36.0	10.0	75.0
	c	51.6	38.1	10.3	43.3
	a	58.5	31.0	10.5	20.0
7	b	54.0	35.0	11.0	14.0
	c	55.3	33.4	11.3	17.5
	a	65.5	24.1	10.4	35.0
8	b	63.0	24.0	13.0	43.0
	c	58.9	25.8	15.3	34.3
	a	51.8	44.8	3.4	7.0
9	b	47.0	46.0	7.0	9.0
	c	49.3	44.9	5.8	5.85
	a	38.3	36.0	25.7	60.0
10	b	38.0	34.0	28.0	93.0
	c	41.5	36.0	22.5	62.8
	a	37.1	36.9	26.0	15.0
11	b	35.0	35.0	30.0	15.0
	c	37.2	34.4	28.4	14.7
	a	55.8	40.1	4.1	76.0
12	b	50.0	40.0	10.0	54.0
	c	51.2	42.4	6.4	72.8
Average values ± 1 SD					
	a	49.1 ± 10.2	34.7 ± 8.2	16.3 ± 13.7	40.2 ± 20.4
	b	46.8 ± 11.0	33.9 ± 7.3	19.3 ± 13.1	43.8 ± 27.5
	c	48.4 ± 10.1	33.3 ± 6.9	18.0 ± 13.2	41.1 ± 20.9
Normal (N = 7)[b]		42.5 ± 5.8	34.5 ± 2.6	23.0 ± 7.4	

[a] All bile samples were obtained from female subjects; samples 1–4 were each obtained from patients with gallstones (radiologically non-visualized gallbladder, sample 4); samples 5–8 from normal women taking oral contraceptives, sample 9 from an infant with celiac disease, and samples 10–12 from normal women on no medication.
[b] Reference #13.

chromatographic use of three group-specific bile acid hydroxysteroid dehydrogenases (HSDH) to rapidly compute the ratios and amounts of the three main bile acids in human biliary extracts. We compare these results with those derived both from GLC and TLC as previously described (4–7).

Downloaded from www.jlr.org by guest, on June 19, 2012

ASBMB JOURNAL OF LIPID RESEARCH JLR

TABLE 2. Correlation coefficients (r values) for comparison of the percentage composition and total bile acid composition

#	Comparison	Cholic	Cheno-deoxycholic	Deoxycholic	Total
			%		
1	A[a] versus B[b]	0.96	0.93	0.99	0.80
2	A versus C[c]	0.91	0.90	0.99	0.98
3	B versus C	0.95	0.96	0.99	0.83

[a] A, direct enzymic assay.
[b] B, gas-liquid chromatography.
[c] C, thin-layer chromatography and enzymic assay.

MATERIALS

3α-Hydroxysteroid dehydrogenase was purchased from Worthington Enzymes, Freehold, NJ. All bile acid standards were obtained from Calbiochemicals, Los Angeles, CA, and β-nicotinamide adenine dinucleotide (NAD) and β-nicotinamide adenine dinucleotide phosphate (NADP) were obtained from Sigma Chemicals, St. Louis, MO.

METHODS

7α- and 12α-Hydroxysteroid dehydrogenases

7α- and 12α-hydroxysteroid dehydrogenases were prepared as described earlier (7, 8). Lyophilized preparations were stored at −20°C.

Extraction of bile-rich duodenal aspirates

Bile-rich duodenal aspirates were collected as described before (9) and extracted according to Folch, Lee, and Sloane Stanley (10) and analyses were performed in duplicate. The combined top phases were evaporated to dryness, reconstituted into a volume of methanol–3% hydrogen peroxide solution 4:1 identical to that of the original aliquot. Aspirates were collected from normal women taking oral contraceptives (for high cholic and low deoxycholic acid proportion), patients with gallstones, a patient with a non-functioning gallbladder (for a high deoxycholic acid proportion), and a patient with celiac disease on tetracycline (for a low deoxycholic acid proportion).

Spectrophotometric assay systems for 3α-, 7α-, and 12-OH groups in biliary extracts

3α-OH Groups. Cuvettes contained 0.9 ml of 1.0 M glycine/NaOH buffer pH 9.5, 50 μl of 53 mM NAD, 1–10 μl of extracted bile acids in solvent (10), and 20 μl of freshly prepared *P. testosteroni* 3α-HSDH (4 mg/ml distilled water). All constituents (except the enzyme) were introduced into the cuvette, mixed thoroughly, and read at 340 nm in a Beckman DB-GT spectrophotometer equipped with a 10 in recorder. The enzyme preparation was introduced subsequently, and the cuvette was monitored until no further absorbance could be measured; the total absorbance at 340 nm was recorded. Total 3α-OH groups were computed on the basis of the $^{\epsilon}$NADH = 6.2×10^3 (11). All assays were performed in duplicate.

7α-OH Groups. The assay system described for 3α-OH groups was utilized except that 10 μl of a solution of lyophilized *E. coli* 7α-HSDH (5 mg/ml distilled water) was used instead of 3α-HSDH.

12α-OH Groups. The assay system described for 3α-OH groups was utilized except that 20 μl of a solution of lyophilized *Clostridium group P* strain C48-50 12α-HSDH (10 mg/ml) was used instead of 3α-HSDH, and NADP was used instead of NAD.

TABLE 3. Recovery of bile acid standards on addition to sample in cuvette and standard bile acid assays

#	Cuvette Contents	Final Absorbance Change at 340 nm			Percent Recovery of Hydroxyl Group on Addition of Standard to Sample		
		3α-HSDH	7α-HSDH	12α-HSDH	3α-HSDH	7α-HSDH	12α-HSDH
1	2.5 μl duodenal bile sample alone	0.254 ± 0.001	0.230 ± 0	0.177 ± 0.002			
2	2.5 μl duodenal bile sample + 10 μl 2 mM cholic	0.380 ± 0.005	0.355 ± 0.002	0.300 ± 0.004	102	101	99
3	2.5 μl duodenal bile sample + 10 μl 2 mM CDC	0.378 ± 0.003	0.353 ± 0.004	0.176 ± 0.003	99	100	
4	2.5 μl duodenal bile sample + 10 μl 2 mM DC	0.378 ± 0	0.230 ± 0.002	0.301 ± 0.002	99		100
5	2.5 μl duodenal bile sample + 10 μl 2 mM LC	0.382 ± 0.004	0.228 ± 0.001	0.177 ± 0.004	102		
	Measured standard bile acids added						
6	10 μl 2 mM cholic	0.124 ± 0.002	0.123 ± 0.002	0.124 ± 0.003			
7	10 μl 2 mM CDC	0.125 ± 0.003	0.124 ± 0	0 ± 0			
8	10 μl 2 mM DC	0.125 ± 0.001	0 ± 0	0.124 ± 0			
9	10 μl 2 mM LC	0.126 ± 0.002	0 ± 0	0 ± 0			

Downloaded from www.jlr.org by guest, on June 19, 2012

ASBMB
JOURNAL OF LIPID RESEARCH

Calculation of the ratio of cholic chenodeoxycholic deoxycholic acids

The ratio of cholic/chenodeoxycholic/deoxycholic acids was computed by solving three simultaneous equations as previously proposed (12):

$$A_{3\alpha} = k((C) + (DC) + (CDC))$$

$$A_{7\alpha} = k((C) + (CDC))$$

$$A_{12\alpha} = k((C) + (DC))$$

where $A_{3\alpha}$, $A_{7\alpha}$, and $A_{12\alpha}$ are the absorbance values of the NAD(P)H produced and (C), (CDC), and (DC) represent the final concentrations of cholic, chenodeoxycholic, and deoxycholic acids. The constant k relates absorbance to the bile acid concentration and is essentially identical to the ϵ value of NAD(P)H which is 6.2×10^3. All analyses were performed under conditions where these reactions go to completion.

Analysis of bile acids by gas–liquid chromatography

The ratio of C/CDC/DC was also determined by gas–liquid chromatography after derivatization of the extracts to the hepatofluorobutyrates (4), and the results were directly compared with both the direct and chromatographic enzymic approaches.

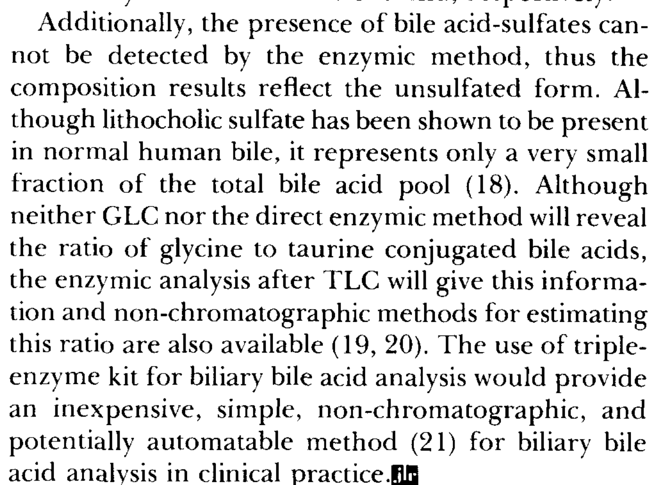
RESULTS AND DISCUSSION

Superimposable standard curves for taurocholate as estimated by each individual enzyme (3α-, 7α-, and 12α-HSDH) are shown in **Fig. 1.** Using 0.1 M glycine/NaOH buffer and in the absence of hydrazine, we were able to achieve complete oxidation of 3α-, 7α-, and 12α-OH groups of both conjugated and unconjugated bile acids with three group-specific enzymes. The bile acid percentage composition as measured by the three methods for all 12 samples is shown in **Table 1;** published values for normal subjects are shown for comparison (13).

There is close agreement among the three methods as shown by comparison of the averages as well as by the correlation coefficients (**Table 2**). As can be seen, the comparison of the ratios determined by the three methods is excellent, with *r* values exceeding 0.90, thus validating the direct approach and the assumption that minor components can be neglected in these particular human bile samples. A comparison of the total bile acid concentration in the sample by GLC gave slightly lower *r* values (Table 2). This result presumably reflects differential losses by the methods.

When a known amount of prestandardized bile acid solutions (cholic, chenodeoxycholic, deoxycholic or lithocholic acid) was added to a cuvette containing an aliquot of duodenal bile sample, the recovery in terms of hydroxyl groups of standard measured was quantitative (**Table 3**). Additionally, the data demonstrate the absolute specificity of the 7α- and 12α-HSDH preparations for their respective hydroxyl groups, as demonstrated before (7, 8, 14–16). It has been shown earlier that both 7α- and 12α-HSDH react with both conjugated and free bile acids (7, 8, 14–16).

Thin-layer chromatography of samples indicated that minor bile acids (lithocholic, ursodeoxycholic and oxo-bile acids) were present only in trace amount (<5%). It must be emphasized that the presence of a disproportionate amount of any of these normally minor components would negate the direct method since it assumes a mixture of the three major bile acids, cholic, chenodeoxycholic, and deoxycholic acid. Thus, in situations such as ursodeoxycholic acid therapy for dissolution of cholesterol gallstones or cholestatic liver disease (17), this direct enzymic approach would not detect the increased amounts of ursodeoxycholic and lithocholic acid, respectively.



Additionally, the presence of bile acid-sulfates cannot be detected by the enzymic method, thus the composition results reflect the unsulfated form. Although lithocholic sulfate has been shown to be present in normal human bile, it represents only a very small fraction of the total bile acid pool (18). Although neither GLC nor the direct enzymic method will reveal the ratio of glycine to taurine conjugated bile acids, the enzymic analysis after TLC will give this information and non-chromatographic methods for estimating this ratio are also available (19, 20). The use of triple-enzyme kit for biliary bile acid analysis would provide an inexpensive, simple, non-chromatographic, and potentially automatable method (21) for biliary bile acid analysis in clinical practice.

This work was supported by grant #MA5075 from the Medical Research Council of Canada and by Dalhousie University Internal Medicine Research Fund. We are indebted to Mrs. Sherry McCarthy for her excellent technical assistance.

Manuscript received 25 June 1979 and in revised form 16 October 1979.

REFERENCES

1. Nair, P. P., and C. Gracia. 1969. A modified gas–liquid chromatographic procedure for the rapid determination of bile acids in biological fluids. *Anal. Biochem.* **29:** 164–171.
2. Ross, P. E., C. R. Pennington, and I. A. D. Boucher. 1977. Gas–liquid chromatographic assay of serum bile acids. *Anal. Biochem.* **80:** 458–465.
3. Sandberg, D. H., J. Sjövall, K. Sjövall, and D. A. Turner. 1965. Measurement of human serum bile acids by gas–liquid chromatography. *J. Lipid Res.* **6:** 182–192.

Downloaded from www.jlr.org by guest, on June 19, 2012

4. Musial, B. C., and C. N. Williams. 1979. Quantitative assay of conjugated and free bile acids as heptafluorobutyrate derivatives by gas–liquid chromatography. *J. Lipid Res.* **20:** 78–85.
5. Shioda, R., P. D. S. Wood, and L. W. Kinsell. 1969. Determination of individual conjugated bile acids in human bile. *J. Lipid Res.* **10:** 546–554.
6. Turnberg, L. A., and A. Anthony-Mote. 1969. The quantitative determination of bile salts in bile using thin-layer chromatography and 3α-hydroxysteroid dehydrogenase. *Clin. Chim. Acta.* **24:** 253–259.
7. Macdonald, I. A., C. N. Williams, and D. E. Mahony. 1975. Lyophylized 7α-hydroxysteroid dehydrogenase: a stable enzyme preparation for routine bile acid analysis. *J. Lipid Res.* **16:** 244–246.
8. Macdonald, I. A., J. F. Jellett, and D. E. Mahony. 1979. 12α-Hydroxysteroid dehydrogenase from *Clostridium group P* strain C48-50 ATCC #29733: partial purification and characterization. *J. Lipid Res.* **20:** 234–239.
9. Williams, C. N., J. W. I. Morse, I. A. Macdonald, R. Kotoor, and M. D. Riding. 1977. Increased lithogenicity of bile on fasting in normal subjects. *Am. J. Dig. Dis.* **22:** 189–194.
10. Folch, H., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissue. *J. Biol. Chem.* **226:** 497–509.
11. Horecher, B. L., and A. Kornberg. 1948. The extinction coefficients of the reduced band of pyridine nucleotides. *J. Biol. Chem.* **175:** 384–390.
12. Macdonald, I. A., C. N. Williams, and D. E. Mahony. 1976. A rapid non-chromatographic analysis of individual bile acids in human bile extracts. *J. Theoret. Biol.* **57:** 385–389.
13. Williams, C.N., I. A. Macdonald, and H. Park-Dincsoy. 1979. Primary bile acid kinetics in patients with primary biliary cirrhosis and in normal subjects. *Clin. Invest. Med.* **2:** 29–40.
14. Macdonald, I. A., C. N. Williams, and D. E. Mahony. 1973. 7α-Hydroxysteroid dehydrogenase from *Escherichia coli:* preliminary studies. *Biochim. Biophys. Acta.* **309:** 243–253.
15. Haselwood, E. S., and G. A. D. Haselwood. 1976. The specificity of a 7α-hydroxysteroid dehydrogenase from *Escherichia coli. Biochem. J.* **156:** 207–210.
16. Mahony, D. E., C. E. Meier, I. A. Macdonald, and L. V. Holdeman. 1977. Bile salt degradation by non-fermentative clostridia. *App. Environ. Microbiol.* **34:** 419–423.
17. Williams, C. N., R. Kay, L. Baker, R. Hurwitz, and J. R. Senior. 1972. Progressive familial cholestatic cirrhosis and bile acid metabolism. *J. Pediat.* **81:** 493–500.
18. Palmer, R. H., and M. G. Bolt. 1971. Bile acid sulfates. I. Synthesis of lithocholic acid sulfates and their identification in human bile. *J. Lipid Res.* **12:** 671–679.
19. Christie, W. H. M., I. A. Macdonald, and C. N. Williams. 1975. Non-chromatographic colorimetric assay for total taurine-conjugated bile acids: application of measurements of glycine:taurine ratio in bile. *J. Lab. Clin. Med.* **85:** 505–514.
20. Hafken-Scheid, J. C. M., and M. P. C. Hectors. 1975. An enzymic method for the determination of the glycine:taurine ratio of conjugated bile acids in bile. *Clin. Chim. Acta.* **65:** 67–74.
21. Domingo, N., J. Amic, and J. Hauton. 1972. Dosage automatique des sels biliares conjugues de la bile par la 3α-hydroxysteroide deshydrogenase. *Clin. Chim. Acta.* **37:** 399–404.

ASBMB JOURNAL OF LIPID RESEARCH

Downloaded from www.jlr.org by guest, on June 19, 2012